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Impaired cognition in rats with cortical dysplasia: additional impact of early-life seizures

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One of the most common and serious co-morbidities in patients with epilepsy is cognitive impairment. While early-life seizures are considered a major cause for cognitive impairment, it is not known whether it is the seizures, the underlying neurological substrate or a combination that has the largest impact on eventual learning and memory. Teasing out the effects of seizures from pre-existing neurological disorder is critical in developing therapeutic strategies. We therefore investigated the additional cognitive effects of seizures on rodents with malformations of cortical development induced with methylazoxymethanol acetate. Pregnant rats were injected with saline or methylazoxymethanol acetate at embryonic Day 15 or 17 to induce differing malformation severity. From the day of birth to 9 days of age, half the pups received 50 flurothyl-induced seizures. All rats underwent testing in the Morris water maze to test spatial memory at 25 days of age (immediate post-weaning) or during adolescence at 45 days of age. Post-weaning rats had severe spatial cognitive deficits in the water maze and seizures worsened performance. In contrast, in animals tested during adolescence, there was no longer an additional adverse effect of seizures. We also investigated whether the severity of the structural abnormality and seizures impacted brain weight, cortical thickness, hippocampal area and cell dispersion area. The mean brain weight in control animals was greater than in rats exposed to methylazoxymethanol acetate at embryonic Day 17, which was greater than rats exposed to methylazoxymethanol acetate at embryonic Day 15. Rats exposed to methylazoxymethanol acetate at embryonic Day 15 had a thinner cortical mantle compared with rats exposed at embryonic Day 17 and control animals. The hippocampal area was similar in rats exposed at embryonic Days 15 and 17 but was smaller compared with controls. Methylazoxymethanol at embryonic Day 17 caused dispersion of the CA1–4 cell layers in the hippocampus, whereas methylazoxymethanol at embryonic Day 15 caused focal nodules in or above the CA1 layer, but the CA1–4 layers were intact and similar to control. Early-life seizures did not have a significant impact on any of these parameters. These observations indicate that the major factor responsible for the cognitive impairment in the rats with cortical dysplasia was the underlying brain substrate, not seizures. These findings have significant implications for the understanding of cognitive impairments in childhood epilepsy and suggest that early aggressive therapy of seizures alone may not be an adequate strategy for minimizing cognitive effects.

Keywords: epilepsy; cognitive impairment; cortical dysplasia; cortical malformations; spatial cognition

Abbreviations: MAM = methylazoxymethanol acetate; ELS = early-life seizures

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Introduction

Childhood epilepsy is associated with a significant risk for cognitive impairments (Farwell *et al.,* 1985; Neyens *et al.,* 1999), which are often severe enough to cause difficulties in school. There are several epilepsy-related factors that predict cognitive impairment including early onset of seizures (Glosser *et al.,* 1997; Bulteau *et al.,* 2000; Bjornaes *et al.,* 2001; Hermann *et al.,* 2002; Cormack *et al.,* 2007), high seizure frequency (Hoie *et al.,* 2006; Hermann *et al.,* 2008), the epilepsy syndrome (Hoie *et al.,* 2006) and length of time during which the child is refractory to treatment (Farwell *et al.,* 1985). These observations have led to the hypothesis that seizures contribute to the cognitive impairments seen in children with epilepsy (Hamiwka and Wirrell, 2009). However, recurrent seizures in humans are extremely unlikely to occur in the absence of an abnormal neural substrate, which can vary from genetic abnormalities to severe structural damage. Remarkably, it is not known whether seizures in the abnormally developing brain confer additional damage. Answering the fundamental question of whether seizures add to the neurological impairment is critical in determining how vigorously seizures are treated in children. If seizures result in additional damage to the already compromised nervous system, then therapy with pharmacological, dietary or surgical therapy aiming to suppress all seizures would be warranted. Conversely, if seizures add little additional impairment to the condition, aggressive therapy would not be necessary.

Most studies examining the relationships between cognitive deficits and seizures in the developing brain have either induced seizures in animals with normal brains or caused an insult to the immature brain following which spontaneous seizures develop. There is extensive evidence that early-life seizures in rodents with normal brains cause moderate impairment in spatial cognition (Karnam *et al.* 2009*a*, *b*). In addition, animals with seizures following a neurological insult also have cognitive impairments (Zhou *et al.,* 2007*b*; Kleen *et al.,* 2010). However, neither approach is entirely clinically relevant as seizures do not spontaneously occur in the normal brain and the latter approach does not allow one to distinguish between the independent effects of aetiology and seizures. This issue can be approached using an animal model with a developmental brain abnormality, but which does not frequently have spontaneous seizures. Inducing early-life seizures in a random subset of these animals will allow a direct investigation of the independent effects of seizures and aetiology.

Malformations of cortical development are present in ~25% of children with intractable epilepsies (Kuzniecky *et al.,* 1993). A well-described model of malformations of cortical development uses prenatal administration of methylazoxymethanol acetate (MAM). MAM is a DNA methylating agent that has been shown to produce malformations of cortical development of varying severities depending on the embryonic day of administration (Cattabeni and Di Luca, 1997). Administration of MAM during critical periods of neuronal development between embryonic Days 15 and 17 causes dramatic disruptions of neuronal migration and the formation of clustered neocortical neurons in the hippocampus (Haddad *et al.,* 1972; Rabe and Haddad, 1972; Paredes *et al.,* 2006). Extensive characterization of the model has shown MAM rats to have cortical and hippocampal malformations similar to those found in humans (Chevassus-Au-Louis *et al.,* 1998), heightened susceptibility to seizures (Baraban and Schwartzkroin, 1996), but relatively few spontaneous seizures (Harrington *et al.,* 2007), pharmacoresistance to anti-epileptic drugs (Smyth *et al.,* 2002) and impaired synaptic plasticity (Ramakers *et al.,* 1993).

We therefore used this well-characterized model of cortical dysplasia to determine whether the neurological substrate upon which seizures occur has any bearing on outcome from seizures. We hypothesized that there is a differential effect of early-life seizures on spatial cognition in rats with a normal and abnormal brain. We report here that in the MAM model of cortical dysplasia, rats have severe cognitive impairment and that seizures confer only a mild and self-limited effect on cognition. In addition we investigated whether cortical thickness, hippocampal area and brain weight predict cognitive outcome. Our data support the contention that the effects of seizures on the normal and abnormal brain are quite different and that the majority of cognitive impairment in rats with cortical dysplasia is due to the abnormal neurological substrate.

Materials and methods

An overview of the experiments performed is shown in Fig. 1 and the number of animals in each experimental group is shown in Table 1.

Animals

All animal procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee, under United States Department of Agriculture and Association for the Assessment and Accreditation of Laboratory Animal Care International-approved conditions, in accordance with National Institutes of Health guidelines. Sprague-Dawley rats were housed with a 12-h light/dark cycle and *ad libitum* access to food and water. Dams were injected intraperitoneally with 20 mg/kg MAM or saline at embryonic Day 15 or 17. Injection of MAM at these time points differentially affects brain development and results in differing degrees of cortical malformations (Haddad *et al.,* 1972; Rabe and Haddad, 1972; Cattabeni and Di Luca, 1997). Twenty-one animals from two litters exposed to MAM at embryonic Day 15, 27 animals from three litters exposed to MAM at embryonic Day 17, and 31 animals from four litters exposed to saline underwent behavioural testing (Table 1).

Flurothyl-induced seizures

It has previously been shown that 50 flurothyl seizures administered from P0 (day of birth) to P10 (10 days of age) in previously normal animals resulted in a significant deficit in spatial cognition when the animals were tested in the Morris water maze (Karnam *et al.,* 2009*a*). In the current study, starting at P0, serial seizures were induced in half of the rat pups by the flurothyl (bis-2,2,2-trifluoroethyl ether) inhalation method previously described in our laboratory (Holmes *et al.,* 1998; Huang *et al.,* 1999; de Rogalski Landrot *et al.,* 2001; Sogawa *et al.,* 2001). For each trial, four to five rats were placed into a plastic chamber (diameter = 13 cm, height = 15 cm). Liquid flurothyl was

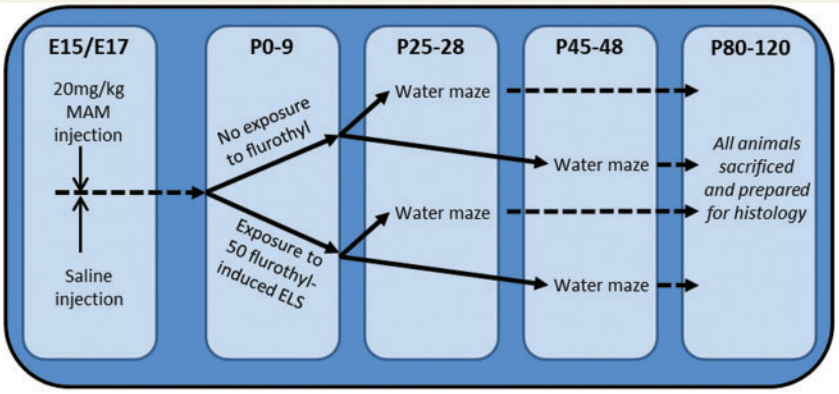

Figure 1 Schematic depiction of the order of experiments. Pregnant dams were injected with either 20 mg/kg MAM or saline at -either embryonic Day 15 (E15) or embryonic Day 17 (E17). At 0–9 days old (P0–9), pups were randomly assigned to no exposure to flurothyl or 50 flurothyl-induced early-life seizures (ELS). Half of these groups were then tested at either 25–28 days old (P25–28) or 45–48 days old (P45–48) in the Morris water maze. All animals were then sacrificed for histological analysis. P80–120 = 80–120 days old. MAM = methylazoxymethanol acetate.

Table 1 Animals, treatments and time of Morris water maze testing

	MAM embryonic Day 15		MAM embryonic Day 17		Control	
	ELS −	ELS +	ELS −	ELS +	ELS −	ELS +
Water maze (immediate post-weaning)	4	5	7	6	8	8
Water maze (adolescent)	6	6	8	6	7	8
Total	10	11	15	12	15	16

ELS = early-life seizures; ELS − = no early-life seizures; ELS + = 50 early-life seizures.

dripped slowly (3 cc/h) onto filter paper in the centre of the container, where it evaporated. Rats were exposed to flurothyl until all of the rats exhibited tonic extension of both the forelimbs and hindlimbs. Seizures lasted 30–60 s following which the animals were removed from the chamber and allowed to recover before being returned to their cages. The time interval between seizures was 60 min. Rats received five seizures per day for 10 days. Control animals were placed in the chamber but not exposed to flurothyl.

Behavioural testing

As there is evidence that early-life seizures in rodents with previously normal brains cause impairments when animals are tested in the Morris water maze (Karnam *et al.*, 2009*a*, *b*), we used the Morris water maze for the current experiments (Morris, 1984). Animals were tested in the Morris water maze either between 25–28 days old or at 45–48 days old. A circular water tank (diameter = 2 m, height = 50 cm) was partially filled with water. Non-toxic white acrylic paint was added to prevent the rat from seeing the submerged platform. A colourless Plexiglass escape platform (10 cm diameter) was positioned 1.5 cm below the water surface in a fixed location in one of the four quadrants for the duration of the experiment. The maze was located in a quiet test room, surrounded by visual cues external to the maze, which were visible from within the pool and could be used by the rats for spatial orientation. Locations of the cues remained unchanged throughout the testing period. The movement of the animals was tracked by an analogue camera located over the centre of the pool and connected to a DVD recorder. On the first day, prior to training, rats were habituated to the pool by allowing them to swim freely for 1 min in the absence of the submerged platform. Then rats had four trials, each from different starting points, per day for 4 days, with each trial having a 120 s ceiling time. After climbing onto the platform, the animal was allowed to remain on the platform for 30 s before commencement of the next trial. In the cases where the animal was unable to find the platform in 120 s, it was guided to the target by the experimenter and was allowed to remain there, or held in place for ~30 s. AnyMaze (version 4.63) software was used to ascertain parameters to be included in the statistical analyses including mean latency to finding the platform, path efficiency, swimming speed and number of body rotations.

Histology

Animals were anaesthetized with urethane (1 g/kg, intraperitoneally) immediately after which rats were perfused transcardially with 120 ml saline followed by 60 ml 4% paraformaldehyde. Brains were extracted from the skull. The olfactory bulbs and cerebellum were cut off and the brains were weighed. Brains were fixed in 4% paraformaldehyde for 2 days and subsequently placed in 30% sucrose solution until they sank to the bottom of the vial. Coronal sections (50 μm) through the entire extent of the hippocampus were performed using a cryostat (Leica CM3050, Leica Microsystems). Every third slice was directly mounted on microscope slides. Sections were then dehydrated in serial ethanol solutions, stained with 0.1% thionin solution (pH 4.0)

for 75 s after which they were rehydrated with ethanol. Slides were then submerged in xylene and coverslipped.

The following measurements were obtained from the histological sections: cortical thickness, hippocampal area, cell dispersion area and nodule area. Figure 4A shows how the cortical thickness, hippocampal area and cell dispersion area were measured. Cortical thickness was a mean measure of both hemispheres, in two consecutive slices, at 0°, 45° and 90° from the horizontal (Fig. 4A). The hippocampal mean area was obtained by tracing the entire hippocampus in both hemispheres in two consecutive slices. The cell dispersion area was obtained by drawing a line between the most lateral parts of the upper and lower blades of the dentate gyrus. A second line was drawn vertical to the most lateral part of the upper blade of the dentate gyrus. The cell dispersion area was then calculated by tracing the area of the cell body layer of the hippocampus between the two lines, one connecting the upper and lower blade of the dentate gyrus and the other vertical line running straight through the hippocampus (Fig. 4B). The mean cell dispersion was obtained for both hippocampi in two consecutive slices. The mean area of the nodule was obtained by tracing the nodule in two consecutive slices. Nodules were found in either left or right hemisphere. Two slices per animal were selected for obtaining all measurements. These were chosen based on the identification of two parameters: the optic tract was as high as the third ventricle and the mammillary bodies were at their largest. This corresponds to the following stereotaxic co-ordinates: 5.70 mm interaural and −3.30 mm from bregma (Paxinos and Watson, 2005). The hippocampal area, cell dispersion area and nodule area were obtained using ImageJ software (NIH; http://rsbweb.nih.gov/ij/).

Statistical analysis

The Morris water maze experiments were evaluated using a multivariable Cox regression time to event approach in STATA Intercooled (10.0; StataCorp). Frailty models were applied in order to deal with the repeated measures. This approach allowed us to investigate the interactions between brain malformations and seizures while accounting for within-animal correlations. A repeated-measures ANOVA was not considered appropriate for these data as they were not normally distributed and the data were censored at 0 and 120 s. The event was defined as finding the platform and the time to event was defined as the time in seconds from placing the animal in the tank to the animal finding the platform. The independent predictor variables tested included day of testing (Days 1, 2, 3 or 4), MAM administration (embryonic Days 17, 15 or control), seizures (yes/no), swimming speed (continuous/variable) and interaction terms.

Other parameters ascertained from the Morris water maze experiments included path efficiency and number of body rotations. These were investigated using generalized estimating equations in Predictive Analytics SoftWare PASW (v.18.0) in order to ensure that the most appropriate data distribution was assumed and in order to deal with repeated measures. The path efficiency data had a gamma distribution and the body rotation data are counts and had a Poisson distribution. The same dependent variables as for the Cox analyses were tested. Analysis of the histological data was performed with an ANOVA in which the effect of group and seizures on the mean histological measurements was established.

Results

A total of 79 rats, from six litters, were included in these experiments. The numbers of animals exposed to MAM administration and early-life seizures are shown in Table 1. Animals with and without MAM-induced cortical dysplasia were either exposed or not exposed to 50 early-life seizures. Animals were then tested at 25–28 days old or 45–48 days old in the Morris water maze. All animals were then sacrificed and the brain was removed for histological evaluation.

Water maze (25–28 days old)

In the immature animals, the time to finding the platform was significantly predicted by the day of testing, whether MAM had been administered, and whether the animals had been exposed to early-life seizures and swimming speed ($P < 0.001$ for all parameters). Compared with the first day of testing, animals were likely to find the platform more quickly on Day 2 [Hazard Ratio (HR) HR 1.8; 95% confidence interval (CI) 1.3–2.6; $P < 0.001$]; Day 3 (HR 2.7; 95% CI 1.9–3.8; $P < 0.001$); and on Day 4 (HR 3.1; 95% CI 2.2–4.3; $P < 0.001$) (Fig. 2A). Control animals were 1.8 times (95% CI 1.2–2.5) more likely to find the platform sooner than rats exposed to MAM at embryonic Day 15 ($P = 0.002$) and 2.1 times (95% CI 1.6–2.9) more likely to find the platform sooner than rats exposed to MAM at embryonic Day 17 ($P < 0.001$) (Fig. 2B). Independently of the effect of MAM, animals not exposed to seizures were 2.3 times (95% CI 1.6–3.5) more likely to find the platform sooner than rats that had 50 early-life seizures ($P < 0.001$, Fig. 2B). The rate of improvement (averaged across the four trials per day) in finding the platform as a function of day of testing was slower in animals exposed to seizures but not to MAM (seizure*day interaction; $P < 0.001$). However, the rates of improvement over consecutive days were no different in the animals exposed to MAM or to a combination of MAM and seizures when compared with control animals.

We also investigated differences in path efficiency and the number of body rotations during the task. Path efficiency represents an index of efficiency of the path taken by the animal to get from the first position to the last. A value of 1 indicates perfect efficiency (i.e. a straight line) and a lower value indicates decreasing efficiency. The body rotation is a measure of the number of times the animal's body completed an entire rotation of 360°. Animals that know where the platform is would be expected to have fewer body rotations.

Overall, rats not exposed to MAM had a better path efficiency than embryonic Day 15 ($P < 0.001$) and embryonic Day 17 ($P < 0.001$; Fig. 2C) MAM rats. Seizure rats were less efficient than those not exposed to seizures ($P < 0.001$; Fig. 2D). There was no difference in mean speed in embryonic Days 15 and 17 MAM and control animals. However, in individual animals, path efficiency was also predicted by swimming speed ($P < 0.001$), i.e. the animals that swam faster were also those that were more efficient at finding the platform. Related to path efficiency is the number of rotations of an animal's body during the swim. Animals exposed to MAM at embryonic Day 17 had more body rotations than animals not exposed to MAM or MAM at embryonic Day 15 ($P < 0.001$; Fig. 2E). Seizure rats had more rotations than those not exposed to seizures ($P = 0.013$; Fig. 2F) and the animals with faster swimming speeds had fewer rotations ($P = 0.014$), independently of whether they were treated with

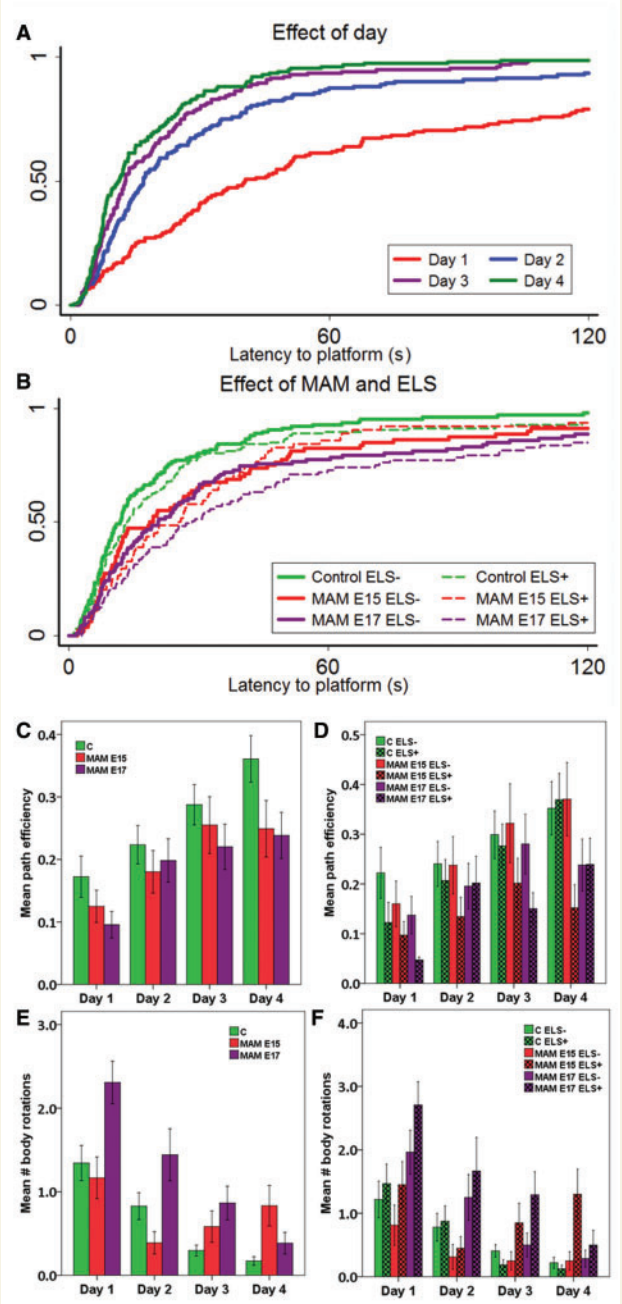

Figure 2 Morris Water maze performed at 25–28 days old. (**A** and **B**) Latency to platform. The *y*-axis is the proportion of animals that have reached the platform. The *x*-axis is the latency to platform in seconds. (**A**) The effect of day across all experimental groups. All groups improved in latency to platform over four consecutive days. For example, on Day 1, 75% of animals found the platform within 120 s. On Day 4, 75% of the animals found the platform within 25 s. This finding was independent of whether the animals had been exposed to MAM or to early-life seizures (ELS). (**B**) The effect of MAM and ELS. Animals exposed to MAM at embryonic Day 15 (E15) and embryonic Day 17 (E17) were significantly ($P < 0.001$) slower at finding the platform compared with controls, independently of whether they received ELS. Animals with ELS were significantly ($P < 0.001$) slower at finding the platform compared with those with no ELS regardless of whether they were control or exposed to MAM at embryonic Day 15 or 17. (**C**, **D**) Path efficiency. (**C**) MAM animals had significantly lower path efficiency compared with controls ($P < 0.001$). (**D**) Animals with ELS had significantly lower path efficiency compared with animals with no ELS ($P < 0.001$). (**E**, **F**) Number of body rotations. (**E**) Animals exposed to MAM at embryonic Day 17 had more body rotations than animals not exposed to MAM or MAM at embryonic Day 15 ($P < 0.001$). (**F**) The number of body rotations was significantly increased in animals with ELS ($P = 0.013$). Error bars = standard error. ELS = early-life seizures.

MAM. In summary, animals exposed to MAM at embryonic Day 15 or 17 had longer latencies to find the platform, lower path efficiency and higher number of body rotations when tested at 25 days old. Additional early-life seizures worsened both MAM-treated and control animal's performance in all these parameters.

Water maze (45–48 days old)

In the adolescent animals, the time to finding the platform was significantly predicted by the day of testing, whether MAM had been administered and swimming speed (all parameters, $P < 0.001$). Compared with the first day of testing, animals were likely to find the platform sooner on Day 2 (HR 2.7; 95% CI 2.1–3.5; $P < 0.001$); Day 3 (HR 4.1; 95% CI 3.2–5.3; $P < 0.001$); and on Day 4 (HR 6.0; 95% CI 4.6–7.6; $P < 0.001$) (Fig. 3A). Control animals were 3.5 times (95% CI 1.6–7.6) more likely to find the platform sooner than rats exposed to MAM at embryonic Day 15 ($P < 0.001$) and 3.6 times (95% CI; 1.3 to 9.0) more likely to find the platform sooner than rats exposed to MAM at embryonic Day 17 ($P < 0.001$; Fig. 3B). There was no significant impact of seizures when the animals reached adolescence ($P > 0.05$; Fig. 3B).

We also investigated differences in the path efficiency to finding the platform and the number of body rotations during the swim. When compared with animals not exposed to MAM, those exposed at embryonic Days 15 and 17 had worse path efficiencies ($P < 0.001$; Fig. 3C) but there was no effect of having had seizures (Fig. 3D). Path efficiency was also predicted by swimming speed ($P < 0.001$). Animals exposed to MAM at embryonic Days 15 and 17 also had more body rotations than animals not exposed to MAM ($P < 0.001$ for both; Fig. 3E) but again there was no effect of seizures ($P > 0.05$; Fig. 3F). The animals with faster swimming speeds had fewer rotations ($P = 0.014$).

In summary, animals exposed to MAM at embryonic Days 15 and 17 had higher latency to platform, lower path efficiency and higher number of body rotations when tested at 45 days old. Importantly, at this age there was no longer an additional effect of seizures identified.

Histology

It is known that animals exposed to MAM have smaller brains than controls (Matsumoto *et al.*, 1972; Balduini *et al.*, 1986). To corroborate this finding we measured brain weight, cortical thickness, hippocampal area and cell dispersion area within the

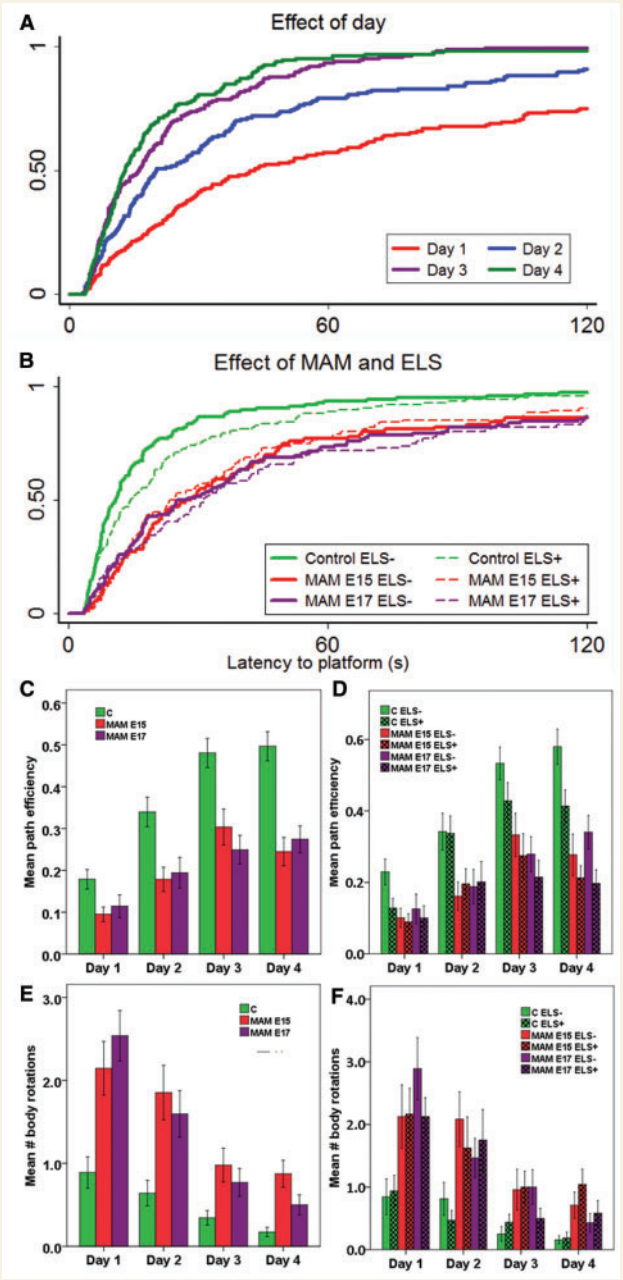

Figure 3 Morris Water maze performed at 45–48 days old. **(A–C)** Latency to platform. The *y*-axis is the proportion of animals that reached the platform. The *x*-axis is the latency to platform in seconds. (**A**) The effect of day across all experimental groups. All groups improved in latency to platform over four consecutive days. For example, on Day 1, 70% of animals found the platform within 25 s. On Day 4, 70% of animals found the platform within 25 s. This was independent of whether the animals had been exposed to MAM or to early-life seizures (ELS). (**B**) The effect of MAM and ELS. Animals exposed to MAM at embryonic Day 15 (E15) and embryonic Day 17 (E17) were significantly ($P < 0.001$) slower at finding the platform compared with controls, independently of whether they received ELS. There was no difference in latency to platform between animals with ELS and those without ELS, regardless of whether they were control animals or had been exposed to MAM at embryonic Day 15 or 17. (**C**, **D**) Path efficiency. (**C**) MAM animals had significantly lower path efficiency compared with controls ($P < 0.001$). (**D**) There was no difference in path efficiency between animals with ELS and those without ELS ($P > 0.05$). (**E**, **F**) Number of body rotations. (**E**) Animals exposed to MAM had significantly greater number of body rotations compared with controls ($P < 0.001$). (**F**) The number of body rotations was no different between animals with ELS and no ELS. Error bars = standard error. ELS = early-life seizures.

CA1, 2, 3 and 4 layers to determine how these alterations in the abnormal brain might affect cognitive outcomes when tested in the Morris water maze.

The mean brain weight in control animals was 1.46 ± 0.002 g compared with 1.003 ± 0.03 g in animals exposed to MAM at embryonic Day 15 ($P < 0.001$) and 1.26 ± 0.003 g in animals exposed to MAM at embryonic Day 17 ($P < 0.001$). The mean brain weight of control animals that did and did not receive seizures was 1.34 ± 0.019 g ($n = 16$) and 1.54 ± 0.031 g ($n = 15$) ($P < 0.05$), respectively. There was no difference between seizure and no seizure in either embryonic Day 15 or 17 MAM animals (Fig. 4E).

Cortical thickness

The mean cortical thickness in animals exposed to MAM at embryonic Day 15 was 1198 ± 57 μm ($n = 15$), which was significantly thinner than the cortex measured in control animals (1541 ± 56 μm; $n = 21$, $P < 0.001$) and in embryonic Day 17 animals (1517 ± 6 μm; $n = 17$, $P < 0.001$). There was no significant difference between control and embryonic Day 17 animals (Fig. 4G).

Hippocampal area, cell layer dispersion and nodule

The mean hippocampal area in control animals was 41.7×10^5 ($\pm 1.4 \times 10^5$ μm^2), which was larger than the hippocampal area in animals exposed to MAM at embryonic Day 15 ($32.1 \times 10^5 \pm 1.1 \times 10^5$ μm^2, $P = 0.001$) and those exposed to MAM at embryonic Day 17 ($30.6 \times 10^5 \pm 1.2 \times 10^5$ μm^2, $P = 0.001$). There was no difference in hippocampal area when the MAM-exposed groups were compared (Fig. 4F).

Injection with MAM during gestation has been shown to result in different types of impaired neuronal migration (Cattabeni and Di Luca, 1997). Two parameters observed in the hippocampus to distinguish between MAM injection at embryonic Days 15 and 17 were cell body layer dispersion in the CA1–4 layers and the mean area of nodules within the same area. Mean cell dispersion area was 3.8×10^5 ($\pm 2.8 \times 10^4$ μm) in animals exposed to MAM at embryonic Day 17. This was significantly larger than cell dispersion area in animals exposed to MAM at embryonic Day 15 ($2.7 \times 10^5 \pm 1.7 \times 10^4$ μm, $P < 0.001$) and in controls ($2.9 \times 10^5 \pm 1.5 \times 10^4$ μm, $P < 0.001$) (Fig. 4H). Nodules were identified in 40% of animals exposed to MAM at embryonic Day 15, and in no hippocampi from the embryonic Day 17 and

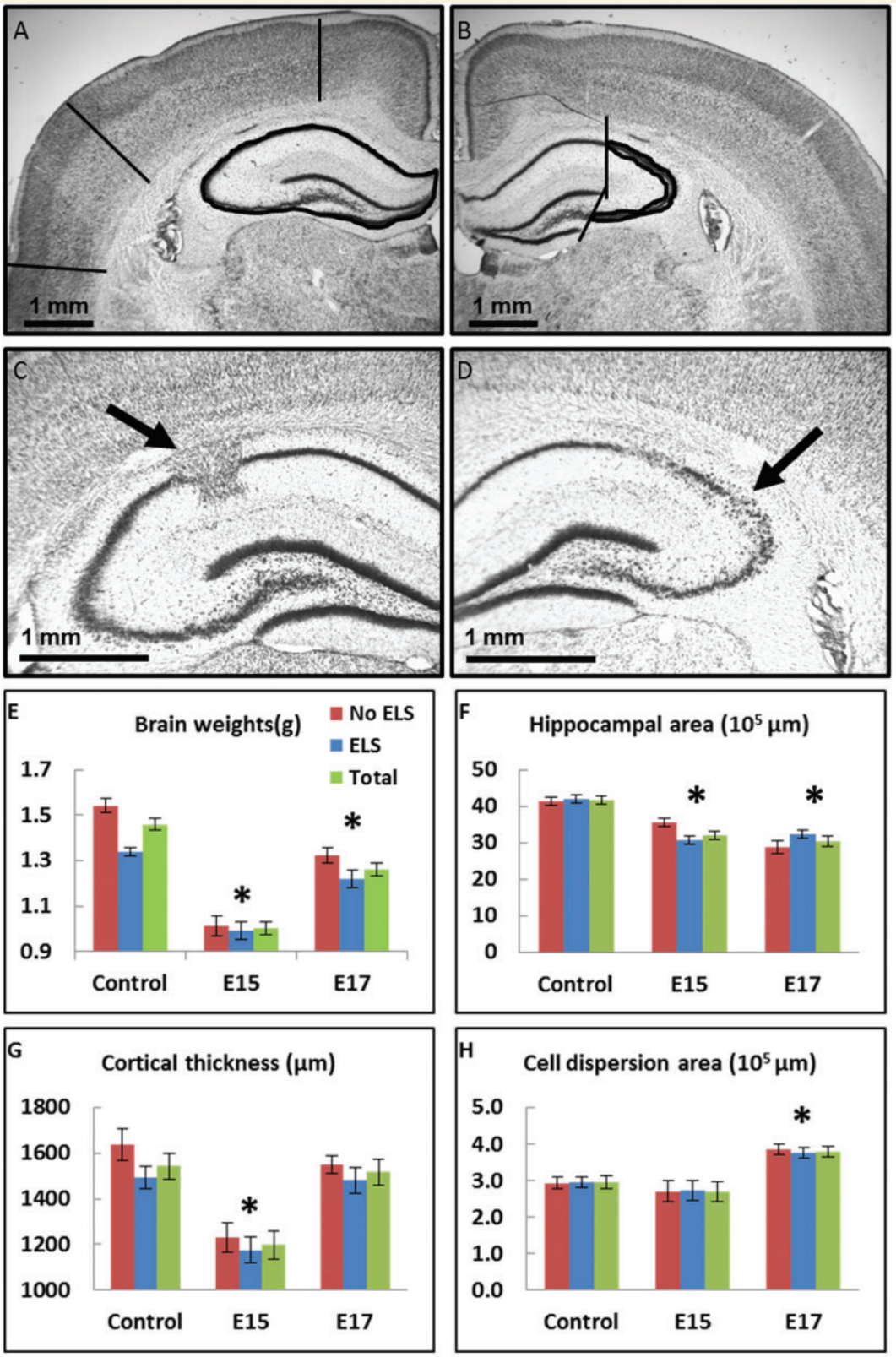

Figure 4 MAM-induced cortical dysplasia animals have abnormal brains. (**A**, **B**) Control sections indicating where cortical thickness, hippocampal area and cell dispersion area were measured. (**C**) Embryonic Day 15 (E15) MAM brain, arrow indicates a nodule. (**D**) Embryonic Day 17 (E17) MAM brain, arrow indicates CA1 cell layer dispersion. (**E**) Mean brain weights (g). (**F**) Mean hippocampal area ($\times 10^5$ µm). Mean was obtained by measuring the left and right hippocampus on two slices. (**G**) Mean cortical thickness (µm). The mean was obtained by averaging 12 measurements; three measurements at 0°, 45° and 90° from the horizontal, in left and right cortices for two consecutive slices. (**H**) Mean cell dispersion area ($\times 10^5$ µm). The area was obtained by drawing a line between the most lateral parts of the

Continued

control groups. The observed nodules had a mean area of 1.8×10^5 ($\pm 2.6 \times 10^4 \mu m^2$).

In summary, embryonic Days 15 and 17 brains were significantly smaller than control brains and also significantly different in weight from each other. Embryonic Day 15 brains had a thinner cortex compared with both embryonic Day 17 and control brains. The hippocampal area was similar in embryonic Days 15 and 17 and significantly smaller compared with controls. Embryonic Days 15 and 17 exhibited different types of dysplasia. Embryonic Day 17 brains had greater dispersed CA1–4 layers in the hippocampus. The embryonic Day 15 brains had focal nodules in or above the CA1 layer, but the CA1–4 layers themselves were intact and similar to control. Importantly, in the brain structures measured here, seizures had no additional effect in either the embryonic Day 15 or 17 groups.

Discussion

The key findings from this study are that severe malformations of cortical development have a major impact on spatial navigation abilities in animals tested in both the immediate post-weaning period and during adolescence. In addition, early-life seizures have a transient impact on spatial learning during development but which are no longer present during adolescence. Thus the major factor responsible for long-term cognitive impairment in the rats with MAM-induced cortical dysplasia was the underlying brain substrate, not seizures. These findings have major implications for the understanding of cognitive impairments in childhood epilepsy and suggest that early aggressive therapy of seizures alone may not represent an adequate strategy for minimizing cognitive effects.

Malformations of cortical development and cognitive impairments are very common in children with epilepsy. To begin to disentangle the effects of underlying brain disease and seizures we established a rodent model of malformations of cortical development, achieved through the injection of MAM, a DNA methylating agent (Matsumoto and Higa, 1966). This model has a low rate of spontaneous seizures (Harrington *et al.,* 2007), and MAM rats were randomly assigned to receive flurothyl treatment. Therefore, the putative occurrence of spontaneous seizures caused by MAM administration could not account for our findings.

The nature of the malformations of cortical development induced by MAM is dependent upon the age at which MAM is administered, and therefore we chose to investigate the effects of MAM administration at two different embryonic ages. The severe reductions in brain weight in animals exposed to MAM at embryonic Day 15 suggest that the malformation extends beyond the hippocampus and cortex and involved subcortical structures, as has been previously shown (Cattabeni and Di Luca, 1997). Although MAM administration at embryonic Day 15 resulted in smaller brain weights than at embryonic Day 17, the spatial impairments were similar in both groups suggesting that any subcortical abnormality in the embryonic Day 15 animals did not have a major impact on these animals' abilities in the Morris water maze. Importantly, spatial performance in MAM rats was considerably decreased when compared with controls and this effect was independent of whether the animals had seizures or not. These effects were seen both in the immediate post-weaning phase and in adolescence. Therefore, the adverse effects of MAM-induced malformations of cortical development were robust and permanent.

The additional impact of early-life seizures on animals with a malformation of cortical development was only observed in animals when they were tested in the immediate post-weaning period and not in adolescent animals. This effect appeared to be smaller than the effect of MAM. The first possible explanation for this finding is that seizures slow normal development, but do not have a permanent adverse effect on the networks underlying spatial learning and memory. This possibility suggests that early successful treatment of seizures may allow networks to rapidly return to a normal developmental trajectory. However, the adult place-cell abnormalities caused by early-life seizures (Liu *et al.,* 2003; Zhou *et al.,* 2007*a*, *b*; Dube *et al.,* 2009; Karnam *et al.,* 2009*b*; Shatskikh *et al.,* 2009) remarkably resemble the place-cell impairments found in immature rats (Scott *et al.,* 2010), supporting the view that seizures retard normal developmental processes. A second possibility is that the seizures lead to permanent disruptions of the normal networks, but that there are compensatory mechanisms that allow adolescent and adult animals exposed to seizures to function similarly to those animals not exposed to seizures. MAM swimming speed, body rotations and path efficiency in the Morris water maze did not differ in rats with and without seizures. This suggests that seizure rats did not use alternative (i.e. non-spatial) strategies to perform the task. It is also possible that the behavioural impairment caused by MAM exposure was too great to allow identification of a more subtle effect of seizures, i.e. a floor effect. This is unlikely as the MAM animals' performance showed day-to-day improvements at a similar rate to the controls. If seizures had an effect, this improvement would not have been observed.

We also carried out histological measurements to assess the relationships between structural brain abnormalities and spatial behaviour. The animals injected with MAM at embryonic Day 15 had smaller brains, thinner cortices and smaller hippocampal areas. Nodules disrupting the hippocampal cell layer were identified, but the cells outside of the nodule seemed to be normally distributed. Despite having similar behavioural impairments, the

Figure 4 Continued
upper and lower blades of the dentate gyrus. A second line was drawn vertical to the most lateral part of the upper blade of the dentate gyrus. The cell dispersion area was then calculated by tracing the area of the cell body layer of the hippocampus between the two lines, one connecting the upper and lower blade of the dentate gyrus and the other vertical line running straight through the hippocampus (**A**). The mean cell dispersion was averaged across both hippocampi in two consecutive slices. Error bars = standard error. $^*P < 0.001$. ELS = early-life seizures.

nature of the histological abnormalities in the animals exposed to MAM at embryonic Day 17 were somewhat different to those identified in animals exposed to MAM at embryonic Day 15. Although the embryonic Day 17 animals had lighter brains than controls they did not have a reduction in cortical thickness. They did have similar reductions in hippocampal areas as the embryonic Day 15 animals. However, in the embryonic Day 17 animals there were no nodules. The predominant histological feature in the embryonic Day 17 animals was the dispersion of the pyramidal cell layer. Given that the Morris water maze is a hippocampal-dependent task, it is not surprising that the two groups of animals exposed to MAM were similarly impaired as both groups had similar reductions in hippocampal area. It is, however, interesting to note that the nature of the hippocampal abnormalities was different in the two MAM-exposed groups. This suggests that a wide range of disruptions to hippocampal neural networks subserving spatial cognition can have important behavioural consequences. As we did not carry out any tasks that are dependent upon neocortical networks it remains uncertain whether the animals exposed to MAM at embryonic Day 15 would have impairments across more cognitive domains than those exposed at embryonic Day 17. Furthermore, there was a significant reduction in brain weight in animals not exposed to MAM but that had early-life seizures. This indicates that seizures differentially affect normal and abnormal brain.

In the current study, we have shown that seizures have little additional impact on cognitive outcomes in spatial learning in animals with severe brain malformations. It remains uncertain whether the interaction between a greater number of seizures and less severe brain abnormalities will be similar. Nevertheless, there are many children with major brain abnormalities who currently receive treatment with multiple anti-epileptic drugs (Holmes, 2002; Meador, 2002) and our findings suggest that the current therapeutic strategies for such children may not be adequate. Although the data from the current study suggest that minimizing seizures with anti-epileptic drugs may have a role in maximizing neural network development, the major predictor of spatial ability was the presence of a malformation of cortical development. However, it is possible that anti-epileptic drugs may not have a positive impact on cognitive impairment. This is supported by a study in which animals exposed to kainic acid-induced status epilepticus and subsequently treated with phenobarbital showed greater memory and learning impairments than animals not receiving phenobarbital (Mikati *et al.,* 1994). A broadening of therapeutic strategies therefore needs to be considered. To this end, environmental enrichment in rodents following weaning has been shown to have beneficial effects including increases in neurogenesis, gliogenesis and synaptogenesis. In addition to these biological effects, environmental enrichment also improves spatial performance in animal models of middle cerebral artery occlusion (Ohlsson and Johansson, 1995).Therefore, therapeutic studies that evaluate the role of environmental enrichment and educational approaches are now essential. In addition, there is a need for research into possible pharmacological enhancement of behavioural therapies. If these strategies are shown to be helpful, then the cognitive abilities of many children with epilepsy could be improved.

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